Enzyme-Linked Immunosorbent Assay of Mycotoxins Using Nylon Bead and Terasaki Plate Solid Phases

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(Received for publication July 29, 1983)

ABSTRACT

Nylon beads and Terasaki plates were tested as solid phases for the enzyme-linked immunosorbent assay (ELISA) of the mycotoxins aflatoxin B1 (AFB1), aflatoxin M1 (AFM1) and T-2 toxin. Both methods had detection limits comparable to that of mycotoxin microtiter plate ELISAs. Using the nylon bead ELISA, ELISA competition curves for AFB1, AFM1 and T-2 toxin exhibited linear response between 1.0 to 100, 0.1 to 100, and 0.1 to 10.0 ng/ml, respectively. Response ranges for Terasaki plate ELISAs of AFB1, AFM1 and T-2 toxin were 1.0 to 50, 0.05 to 0.50, and 0.5 to 1.0 ng/ml, respectively. The new procedures did not require specialized instrumentation and may be used as an economical screening method for mycotoxins in the field and to diagnose certain mycotoxicoses.

Mycotoxins are a group of secondary fungal metabolites that can be toxic to humans or animals after ingestion or exposure (2). Since these compounds are known to occur naturally in agricultural commodities, such as corn, wheat, nuts, milk and animal feedstuffs, there has been increased interest in the development of rapid, sensitive techniques for their detection in foods, feeds and biological fluids.

Previous investigations by our laboratory and others have described the suitability of competitive enzyme-linked immunosorbent assays (ELISAs) for monitoring aflatoxin B1 (AFB1), (1,5,9,13), aflatoxin B2a (AFB2a) (6,14), aflatoxin M1 (AFM1) (16), T-2 toxin (12,15) and ochratoxin A (17). The assays are conducted by simultaneous incubation of standard or sample mycotoxin with mycotoxin-horseradish peroxidase conjugate over a specific solid-phase antibody. The solid phase is washed and total bound peroxidase determined. Standard competition curves of log10 mycotoxin concentration vs. absorbance are then prepared for sample determination. Analysis of food and feeds involves a solvent extraction, dilution of the extract in buffer, and direct analysis by ELISA (5,14). In the case of milk, ELISAs for aflatoxin M1 are performed directly on the sample (16). The ELISAs are generally more sensitive, simpler and faster than conventional chemical methods or radioimmunoassay for the quantitative analysis of mycotoxins.

In many cases, a simple qualitative screening assay for mycotoxins at a threshold level may be desirable for field analysis and diagnosis of mycotoxicoses. However, previously described mycotoxin ELISAs employ the 96-well polystyrene microtiter plate as the solid phase for attachment of specific antibody. A major disadvantage with this solid phase is the cost of accessory equipment required for washing the microtiter plates and making spectrophotometric determinations. Two alternative solid phases used for the ELISA of protein antigens that may be applicable to mycotoxin analysis are the nylon bead and Terasaki plate. Hendry and Herman (8) first described a procedure where antibodies are immobilized to nylon beads. Figure 1 demonstrates how immunoglobulins and other proteins can be covalently linked to partially hydrolyzed nylon by use of glutaraldehyde. The procedure is efficient and ELISAs for protein antigens using nylon compare favorably with other solid phases. Nylon bead ELISAs do not require a special washing apparatus and the solid-phase can be readily separated from the enzymatic end-product for absorbance measurement on a standard spectrophotometer. Paterakiet al. (11) utilized Terasaki microtissue culture plates in the ELISA of protein antigens. These plates contain 60 10-µL conical wells which allow for economical use of reagents and easy washing between steps. End-product absorbance is qualitatively measured by visual estimation.

The objectives of the present investigation were twofold: (a) to develop optimal ELISA procedures for screening of mycotoxins using nylon bead and Terasaki plate solid phases and (b) to compare these new methods to microtiter plate procedures on the basis of sensitivity.

MATERIALS AND METHODS

Chemicals

All inorganic chemicals and organic solvents were of reagent grade quality. Horseradish peroxidase (type VI), RIA-grade bovine serum albumin...
groups. The latter are conjugated to immunoglobulin (R-NH₂) with partial hydrolysis of nylon results in free carboxyl and amino attachment of immunoglobulin to nylon bead solid phase. The functional reagent glutaraldehyde.

of the University of Wisconsin. Stock mycotoxin standards (10^6/ml) were handled with vinyl gloves in a fume hood. Contaminated plastic ball (Precision Ball Co., Chicago, IL) were gently shaken in 100 ml of 3.5 M HCl for 16 h at 25°C. These were decanted and washed three times with distilled water. The beads were gently shaken in 8% glutaraldehyde (Fisher Scientific, biological grade) for 90 min at 25°C, then decanted and washed in distilled water three more times. They were stored in distilled water at 4°C for a maximum of 2 months.

Purified antibody was diluted between 1 to 25 μg/ml in 0.1 M phosphate-buffered, pH 7.5, normal saline (PBS) and added to treated beads (0.5 ml/bead). Beads were gently shaken for 60 min at 25°C and then aspirated. PBS containing 1.0% (vol/vol) BSA (PBS-BSA) was added to the beads (0.5 ml/bead) and mixed for an additional 60 min at 25°C. PBS-BSA was aspirated off and the beads washed four times with PBS containing 0.05% Tween 20 (PBS-Tween).

For ELISA, individual beads were placed in glass tubes (13 x 100mm) and incubated with 50 μl of mycotoxin standard in PBS and 50 μl of mycotoxin peroxidase (5 to 50 μg/ml) in PBS-BSA, and gently shaken for 60 min at 25°C. The beads were then washed five times by repeated addition of 1.0 ml of PBS-Tween, gentle vortexing and aspiration. To determine bound horseradish peroxidase activity, 1.0 ml of ABTS substrate (10) was added, and the tubes gently mixed for 60 min at 25°C. Hydrofluoric acid-EDTA stopping reagent (1.0 ml; 10) was added and absorbance at 414 nm determined by a Beckman DU spectrophotometer. Competition curves were prepared by plotting log₁₀ mycotoxin concentration vs. absorbance.

Terasaki plate ELISA
A modification of the microtiter plate method of Pestka et al. (13) was used for preparation of the Terasaki plate solid phase. Mycotoxin antiserum was diluted in PBS (5 to 50 μg/ml) and 10 μl added to wells of Falcon 3034 Microtest Terasaki tissue culture plates (Becton Dickinson and Co., Cockeysville, MD). These were dried under a hair dryer (40°C). To minimize non-specific binding, 0.25 ml of PBS-BSA was added, and plates were incubated for 15 to 30 min at 37°C. Plates were decanted and washed four times by (a) filling with PBS-Tween, (b) covering, (c) vigorously shaking and (d) decanting. Wells were aspirated then incubated with 5 μl of standards in PBS and 5 μl of mycotoxin-peroxidase (5 to 50 μg/ml) in PBS-BSA for 60 min at 37°C with the cover in place. Wells are aspirated, washed four more times, aspirated again, and then rapidly filled with 10 μl of ABTS substrate. Stopping reagent was not used in this procedure. After 30 to 60 min of incubation at 37°C, green-colored end-product in wells was qualitatively read over an opaque light source.

Microtiter plate ELISA
The BSA-glutaraldehyde method described by Pestka et al. (12) was used for the attachment of antibody to the 96-well microtiter plate solid phase. A modification of previous ELISA procedures (13-16) designed to decrease nonspecific peroxidase binding was used for mycotoxin analysis. Briefly, antibody-coated wells were initially washed three times with filling with 0.25 ml PBS-Tween and aspirating with a Dynatech Mini Wash (Dynatech Laboratories, Alexandria, VA). Wells were then simultaneously incubated with 25 μl of mycotoxin standard in PBS and 25 μl of mycotoxin-peroxidase (5 to 50 μg/ml) in PBS-BSA for 60 min at 37°C with covers in place. To minimize nonspecific peroxidase binding, plates were washed six times by sequential addition and aspiration of the following volumes of PBS-Tween: 0.05, 0.10, 0.15, 0.20, 0.25 and 0.25 ml. After the final aspiration, total bound enzyme was determined with ABTS substrate as described previously (2). Absorbance for each well was determined on a Dynatech Mini ELISA reader and plotted against log₁₀ standard mycotoxin concentration for preparation of a competition curve.

RESULTS AND DISCUSSION
The methods described here for nylon bead, Terasaki plate and microtiter plate mycotoxin ELISAs represent optimal conditions determined by preliminary experiments. Typical competition curves for the three mycotoxins in the nylon bead ELISA are illustrated in Figure 2. AFB₁ antiserum was used because it is specific for several aflatoxins and had been used previously to quantitate AFB₁ (6,14). Minimum sensitivity for AFB₁ detection in the nylon bead ELISA using AFB₁ peroxidase and AFB₂₉ antiserum was 1 ng/ml. Nylon bead ELISAs for AFM₁ and T-2 toxin were relatively more...
sensitive and could detect as little as 0.1 ng of the two mycotoxins per ml.

Using visual estimates, minimum sensitivities for AFB₁, AFM₁ and T-2 toxin in the Terasaki plate ELISAs were 1.0, 0.05 and 0.5 ng/ml, respectively. Sensitivity for AFB₁ with the AFB₂α antibody was thus less than that for the other two mycotoxins. Figure 3 is a photograph of a Terasaki plate ELISA for AFM₁ in which standards were added directly to homogenized milk. Each well was precoated with 10 μl of a 3.0-μg solution of AFM₁ antibody per ml. Milk standards were introduced with AFM₁ peroxidase directly into the Terasaki plate without solvent extraction. Results suggested that the Terasaki plate may be a suitable rapid method for determining AFM₁ in dairy products at the current 0.5-ppb FDA action level.

Sensitivities of the antisera and peroxidase conjugates used for nylon bead and Terasaki plate ELISAs were also determined in 96-well microtiter plate ELISAs. As with the two other solid phases, sensitivity for AFB₁ (1.0 ng/ml) was considerably less than that for AFM₁ (0.05 ng/ml) and T-2 toxin (0.1 ng/ml). Greater sensitivity for AFM₁ and T-2 relative to AFB₂α in all three methods is likely to be related to conjugation methods used for raising specific antibodies to these toxins. Since mycotoxins are low molecular weight and not immunogenic, they must be conjugated to a BSA-carrier to obtain specific antibodies. AFM₁ is conjugated via a carboxymethyl oxime spacer and T-2 toxin via a hemisuccinate spacer which allows these compounds to project distally from the carrier protein. In the case of AFB₂α, the toxin is partially degraded by opening the furan ring and is conjugated to BSA by reductive alkylation. The aflatoxin molecule would be oriented close to the carrier protein and would be less immunodominant relative to BSA than the AFM₁-BSA and T-2 toxin-BSA immunogens. AFB₂α-BSA might thus generate antibodies of lower affinity than AFM₁-BSA or T-2-BSA resulting in less sensitivity in the ELISA.

The response ranges (that part of the ELISA competition curve where absorbance decrease is approximately linear) for all three solid phase assays of AFB₁, AFM₁ and T-2 toxin are summarized in Table 1. Response ranges for AFB₁ were approximately the same by all three methods: 1.0 to 100, 1.0 to 50, and 1.0 to 100 ng/ml for the nylon bead, Terasaki plate, and microtiter plate, respectively. However, response ranges for AFM₁ and T-2 were broader for the nylon bead ELISAs and narrower for the Terasaki plate ELISAs system than for the microtiter plate system (Table 1). The latter observation is in agreement with the work of Saunders (18) which indicates ELISA response ranges increase with increasing solid-phase surface area.

**CONCLUSIONS**

Results presented here suggest that both the nylon bead and Terasaki plate ELISAs are suitable for screening mycotoxins and have sensitivities equivalent to those ob-

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**TABLE 1. Summary of ELISA response ranges for various solid phases.**

<table>
<thead>
<tr>
<th>Solid phase</th>
<th>AFB₁ (ng/ml)</th>
<th>AFM₁ (ng/ml)</th>
<th>T-2 toxin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nylon bead</td>
<td>1.0-100</td>
<td>0.1-100</td>
<td>0.1-10.0</td>
</tr>
<tr>
<td>Terasaki plate</td>
<td>1.0-50</td>
<td>0.05-0.5</td>
<td>0.5-1.0</td>
</tr>
<tr>
<td>Microtiter plate</td>
<td>1.0-100</td>
<td>0.05-1.0</td>
<td>0.1-1.0</td>
</tr>
</tbody>
</table>

*That part of competition curve where absorbance decrease was linear relative to increase in log₁₀ mycotoxin concentration.

AFB₂α antiserum and peroxidase-conjugate were used for AFB₁ determination (6).
tained by the microtiter plate system. As was demonstrated by the Terasaki ELISA for AFM$_1$ in milk, these procedures may be readily adaptable to the field analysis of mycotoxins and to the diagnosis of certain mycotoxicoses in humans and animals. The major advantage of the nylon bead solid phase as compared to the microtiter plate is the lack of a requirement for a special washing apparatus and microtiter plate spectrophotometer. A disadvantage is that because of the laboratory manipulations required for nylon bead assays, fewer samples per unit time can be processed than by the microtiter plate approach. Advantages of the Terasaki plate system include economy of reagents, high sample throughput, and the lack of need for washing equipment. However, the procedure is semiquantitative because visual absorbance estimates are made. Laboratories that are developing mycotoxin ELISA systems should therefore take into account the merits of each of the solid phase systems discussed here relative to each laboratory’s specific needs.

ACKNOWLEDGMENTS

This work was supported by the College of Agricultural and Life Sciences, the University of Wisconsin-Madison, by Public Health Service research grant CA15064 from the National Cancer Institute, and by Public Health Service training grant T32 ES07015 from the National Institute of Environmental Health Sciences.

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